Characterization of a spleen sulphotransferase responsible for the 6-O-sulphation of the galactose residue in sialyl-*N*-acetyl-lactosamine sequences

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An enzyme which catalyses the transfer of sulphate from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to C-6 of galactose in the NeuAc α 2-3Gal β 1-4GlcNAc (3'SLN) sequence has been found in rat spleen microsomes and its specificity indicates that it is well suited to participate in the assembly of 3'-sialyl-6'-sulpho-LacNAc [NeuAc α 2-3Gal(6-SO₃) β 1-4GlcNAc] and 3'-sialyl-6'-sulpho-Lewis^x [NeuAc α 2-3Gal(6-SO₃) β 1-4(Fuc α 1-3)GlcNAc] saccharide groups which have been implicated as selectin ligands. This sulphotransferase has a strict requirement for oligosaccharide acceptors which are capped by an α 2-3-linked sialic acid residue, although GlcNAc in 3'SLN can be substituted by Glc, and Gal β 1-4GlcNAc can be replaced by Gal β 1-3GlcNAc without loss of activity. The finding that 3'-sialyl Lewis^x was inert as an acceptor suggested that fucosylation, in contrast with sialylation, follows the addition of the sulphate group. Since fetuin glycopeptides containing the NeuAc α 2-3Gal β 1-4GlcNAc sequence had a similar affinity for the enzyme as the unattached 3'SLN, it would appear that the acceptor determinants reside primarily in the peripheral trisaccharide constellation. The position of the sulphate on C-6 of galactose

was elucidated by Smith periodate oxidation, hydrazine/nitrous acid/NaBH₄ treatment and elder (*Sambucus nigra*) bark lectin chromatography of the desialylated [³⁵S]sulphate-labelled products of the enzyme. Assays carried out with 3'SLN as acceptor indicated that the sulphotransferase had a pH optimum between 6.5 and 7.0 and a dependence on a bivalent cation best met by Mn²⁺ (12–25 mM); Triton X-100 (0.02 to 0.35 %) brought about maximal stimulation. Tentative *K_m* values determined for this enzyme were 4.7 μ M for PAPS, and 0.72 mM and 1.16 mM for 3'SLN and fetuin glycopeptides respectively. A survey of several rat organs indicated that the PAPS:3'SLN-6-O-sulphotransferase is selectively distributed with maximal activity occurring in spleen which was substantially greater than thymus or lymph nodes. In contrast, other enzymes (i.e. PAPS:Gal-3-O- and GlcNAc-6-O-sulphotransferases) involved in the sulphation of sialyl-lactosamine and lactosamine sequences, which in the sulphated form are believed to also be selectin ligands, were more evenly distributed in lymphoid tissues. Relatively high activities for all three enzymes were found in brain.

INTRODUCTION

It has now been well established that N- and O-linked oligosaccharides of a diverse assortment of secreted as well as membrane-associated glycoproteins contain sulphate substituents which have been localized to defined positions on Gal [1–4], GalNAc [3,5], GlcNAc [1,2,4,6–11] and Man [12] residues. Studies in various cells have indicated that the addition of these anionic groups occurs as a late, *trans*-Golgi network-localized event [11,13]. Moreover, a number of specific sulphotransferases have now been characterized, and these include enzymes which attach sulphate to C-3 of Gal [14], C-4 of GalNAc [15] and C-6 of GlcNAc [16].

Although the physiological role of sulphate groups in glycoproteins remains to a large extent undetermined, their presence on the Gal and GlcNAc residues of ligands for selectins appears to provide an intriguing example in which they have been implicated in a major biological process represented by the attachment of leucocytes to endothelial cells (see [17–19] for reviews). A number of binding studies with various oligosaccharides have suggested that sulphate groups may be involved in the carbohydrate-dependent interaction of circulating leucocytes with vascular endothelial cells in lymphoid tissues during the homing phenomenon [20–24]. Furthermore, structural investi-

gations have demonstrated that the carbohydrate chains of GlyCAM-1, an endothelial-derived ligand for L-selectin, contains 6'-sulphated sialyl Lewis^x [NeuAc α 2-3Gal(6-SO₃) β 1-4(Fuc α 1-3)GlcNAc] capping groups [25], which in subsequent studies have been implicated in leucocyte-endothelial cell adhesion [23,24].

The assembly of a NeuAc α 2-3Gal β 1-4GlcNAc (3'SLN) sequence with a sulphate group on C-6 of the galactose residue would appear to require a sulphotransferase distinct from those which have already been described and which, in view of the *in vivo* demonstration that sulphation occurs subsequent to sialylation [11], should act optimally on oligosaccharides containing a terminal neuraminic acid residue. In the present study, we undertook a search in various rat tissues for an enzyme with such a specificity employing 3'SLN as an exogenous acceptor probe. This investigation led us to the finding that 3'-phosphoadenosine 5'-phosphosulphate (PAPS):3'SLN-6-O-sulphotransferase activity is most prominently expressed in spleen. Accordingly, we utilized microsomal membranes from this lymphoid organ to characterize a unique enzyme which appears to be well suited for sulphating galactose at the C-6 position in preassembled NeuAc α 2-3Gal β 1-4GlcNAc sequences. The tissue distribution of this novel sulphotransferase was found to be quite distinct from those of two other enzymes which are believed to participate

Abbreviations used: 3'SLN, 3'-sialyl-*N*-acetyl-lactosamine (NeuAc α 2-3Gal β 1-4GlcNAc); PAPS, 3'-phosphoadenosine 5'-phosphosulphate; LacNAc, *N*-acetyl-lactosamine; SNA, *Sambucus nigra* agglutinin; *R*_{LacH₂}, chromatographic mobility relative to lactitol; AnManH₂, 2,5-anhydromannitol; for brevity the saccharides 3'SL, 6'SL, LSTa, LSTc, 3'SLe^x, 3'S3FL, DSLNT and DSL are defined in the text; -O-Me, methyl glycoside.

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in the sulphation of the *N*-acetyl-lactosamine (LacNAc) and sialyl-LacNAc sequences.

EXPERIMENTAL

Preparation of rat spleen membranes

After an overnight fast, male rats (200–250 g; CD strain; Taconic Inc., Germantown, NY, U.S.A.) were killed by decapitation and their spleens were rapidly excised. This tissue was subsequently cut into small pieces, followed by disruption with a Polytron homogenizer (2×5 s at a setting of 7) in 4 vol. of ice-cold 50 mM Tris/acetate buffer, pH 7.0, containing 25 mM potassium acetate and 0.35 M sucrose. The homogenate was then centrifuged successively in Berkman L5-65 ultracentrifuge at 2000 *g* for 10 min, 12000 *g* for 15 min and 144000 *g* for 45 min. The last pellet, representing the microsomal fraction, was washed with 50 mM Tris/acetate buffer, pH 7.0, containing 25 mM potassium acetate, and then suspended in the same buffer at a protein concentration of approx. 18 mg/ml with a Dounce homogenizer.

Assay of 3'-sialyl-*N*-acetyl-lactosamine sulphotransferase

In the standard assay, the microsomal membranes (90–180 μ g of protein) were incubated with 0.5 μ Ci of 3'-phosphoadenosine 5'-phospho[35 S]sulphate (PAPS; 1.8–2.4 Ci/mmol; DuPont–New England Nuclear) and 50 nmol of 3'SLN (Oxford Glycosystems, Abingdon, Oxon, U.K.) for 60–90 min at 30 °C in 65 μ l of 50 mM Tris/acetate buffer, pH 7.0, containing 100 mM NaF, 2 mM ATP, 25 mM manganese acetate and 0.1 % (v/v) Triton X-100. The reaction was terminated by the addition of 5 μ l of a 100 μ g/ μ l BSA solution, followed by 3 ml of ice-cold 80 % (v/v) ethanol. After mixing, the samples were centrifuged at 600 *g* for 15 min at 2 °C and the protein pellet was then washed twice with 2 ml of ice-cold 80 % (v/v) ethanol. The combined supernatants were dried under a vacuum at 35 °C, dissolved in 3 ml of 0.2 M NaCl and applied to a column of charcoal/Celite [Darco G-60/Celite 535, 1:1 (w/w)] [26] from which, after washing with 10 ml of 0.1 M NaCl and 15 ml of water, the saccharides were eluted with 12 ml of 50 % (v/v) ethanol. TLC of the eluted material was carried out on cellulose-coated plates in solvent system A (see below) and after detection by fluorography, the sulphated saccharide products were quantified by liquid-scintillation counting subsequent to being scraped into vials to which 0.5 ml of water followed by 4 ml of scintillation fluid (Monofluor; National Diagnostics, Manville, N.J., U.S.A.) were added.

Although kinetic studies were carried out under conditions in which less than 1 % of the substrate was utilized, there was a fall-off from linearity with time which made it possible to obtain only approximate K_m values. The values for fetuin glycopeptides and 3'SLN were calculated with the Michaelis–Menten equation, while the K_m for PAPS was determined with the Hill equation. The software for these calculations [27] was kindly made available by Dr. P. J. Brooks (Carleton University, Ottawa, Canada).

For specificity studies, this assay system was used with the following saccharides: 6'-sialyl-*N*-acetyl-lactosamine (6'SLN), 3'-sialyl-lactose (3'SL), 6'-sialyl-lactose (6'SL), NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc (LSTa), NeuAc α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc (LSTc), 3'-sialyl Lewis^x (3'SLe^x), 3'-sialyl-3-fucosyl-lactose (3'S,3FL) and disialyl lacto-*N*-tetraose (DSLNT) from Oxford GlycoSystems; *N*-acetyl-lactosamine (LacNAc) and disialyl-lactose (DSL) from Sigma; and 3'-sialyl Lewis^x methyl glycoside from Toronto Research Chemicals (North York, Ontario, Canada). Fetuin glycopeptides containing either the native or the desialylated N-linked carbohydrate units were prepared from Pronase digests as previously described [14].

When glycopeptides were used as acceptors, their radiolabelled sulphated products were recovered at the origin after TLC in solvent system A.

Determination of tissue distribution of several sulphotransferases

Several tissues from freshly killed overnight-fasted male rats (200–250 g) as well as frozen rat lymph nodes (Pel-Freez Biologicals, Rogers, AR, U.S.A.) were disrupted with a Polytron homogenizer in 5 vol. of buffer in a manner similar to that described for spleen. Postnuclear supernatants (600 *g*, 10 min) of the homogenates were then centrifuged for 60 min at 100000 *g* to obtain membrane pellets which, after two washes with 100 mM Tris/acetate, pH 7.0, containing 25 mM potassium acetate, were suspended in this buffer at a protein concentration of about 20 mg/ml. These postnuclear membranes were then assayed for 3'SLN-6-O-sulphotransferase activity in a manner described above, as well for PAPS:Gal-3-O- and GlcNAc-6-O-sulphotransferase activities, as previously reported [14,16]. These three comparative assays were carried out with 0.5 μ Ci of [35 S]PAPS and 65 nmol of 3'SLN, LacNAc, or GlcNAc β 1-6Man α 1-O-Me acceptors respectively for 90 min at 30 °C in a total volume of 65 μ l (-O-Me is methyl glycoside).

Neuraminidase digestions

Purified [35 S]sulphate-labelled 3'SLN, LSTa and fetuin glycopeptides were incubated with *Arthrobacter ureafaciens* neuraminidase (50 munits) in 50 μ l of 0.15 M sodium acetate, buffer pH 5.0, at 37 °C for 24 h in the presence of toluene. At that time, a further addition of enzyme (25 munits) was made and the incubations were continued for another 24 h. The digestions were terminated by heating, and the radiolabelled products were deproteinized and desalted by adsorption on a charcoal/Celite column from which they were eluted with 50 % (v/v) ethanol as described above.

Structural and analytical procedures

[35 S]Sulphate-labelled fetuin glycopeptides were submitted to hydrazine/HNO₂/NaBH₄ treatment as previously described [1,14], and the radiolabelled products were examined in solvent system B (see below). 3 H-labelled sulphated disaccharide standards were prepared as already reported [14]. Neuraminidase-treated [35 S]3'SLN was allowed to react with sodium metaperiodate as described previously [1]. After NaBH₄ reduction of the product, the sample was desalted on a charcoal/Celite column and submitted to Smith degradation (0.05 M HCl, 80 °C, 1 h) followed by TLC. For the preparation of radiolabelled α -glycerol sulphate standard, the [35 S]GlcNAc(6-SO₄) β 1-6Man α 1-O-Me product of rat liver Golgi PAPS:GlcNAc-6-O-sulphotransferase [16] was submitted to a Smith periodate degradation. The α -glycerol [35 S]sulphate, which is the only radiolabelled product of this reaction, was then isolated by preparative TLC. Protein was quantified by the Bio-Rad binding assay [28], using BSA as a standard.

Elder (*Sambucus nigra*) bark lectin (SNA) chromatography

A column (0.7 cm $\times$ 9.0 cm) of agarose-linked SNA (Vector Laboratories, Burlingame, CA, U.S.A.) was equilibrated at 4 °C with 0.15 M NH₄HCO₃ containing 0.1 mM calcium acetate prior to the application of [35 S]sulphate-labelled 3'SLN before and after its desialylation with neuraminidase. After application of the sample, the column was eluted with the equilibration buffer, 1 ml fractions were collected, and their radioactivity was

monitored by liquid-scintillation counting. Other sulphated disaccharides submitted to the lectin chromatography were prepared by sulphotransferase {[³⁵S]Gal(3-SO₄)/β1-4GlcNAc and [³⁵S]GlcNAc(6-SO₄)/β → 1Man} or galactosyltransferase {[³H]Galβ1 → 4GlcNAc(6-SO₄)} action as previously described [14,16].

TLC

Chromatography was performed on plastic sheets precoated with cellulose (0.1 mm thick; Merck) in pyridine/ethyl acetate/water/acetic acid, 6:3:3:1 (by vol.) (solvent system A) or pyridine/ethyl acetate/water/acetic acid, 5:5:3:1 (by vol.) (solvent system B). A wick of Whatman no. 3 paper was clamped to the top of the plate during chromatography; components were revealed by fluorography and quantified by scintillation counting after elution with water. For preparative separations the radioactive saccharides were solubilized with water and the eluates were extracted with peroxide-free diethyl ether to remove scintillants.

Radioactive measurements

Liquid scintillation counting was carried out in Monofluor with a Beckman LS 7500 instrument. Components on thin-layer plates were located by fluorography at -80 °C on X-Omatic AR film (Eastman Kodak) after being sprayed with a scintillation mixture containing 2-methylnaphthalene [29].

RESULTS

Assay of spleen sulphotransferase activity towards sialylated oligosaccharides

Rat spleen microsomes were observed to effectively catalyse the transfer of sulphate from [³⁵S]PAPS to 3'SLN, although no sulphation of the α2-6-linked isomer (6'SLN) took place (Figure 1). In a similar fashion, incorporation of sulphate into 3'-sialyl-

Figure 1 TLC examination of the products resulting from the action of rat spleen microsomal sulphotransferase on several sialylated saccharides

Incubations with microsomal enzyme (175 µg of protein) were carried out in the presence of the indicated acceptors (50 nmol) or in their absence (—) with 0.5 µCi of [³⁵S]PAPS at 30 °C for 60 min as described in the Experimental section. Subsequent to deproteinization and fractionation on charcoal/Celite columns, equal aliquots of the samples were applied to a cellulose-coated plate for chromatography in solvent system A for 80 h, after which the components were revealed by fluorography. The position to which PAPS migrated is indicated.

Figure 2 Effect of pH on the activity of rat spleen sulphotransferase toward 3'SLN

Incubations were performed at the indicated pH values with microsomal enzyme (138 µg of protein) for 90 min under standard assay conditions. The following buffers were used: sodium acetate, pH 4.0, 4.5 and 5.0; sodium Mes, pH 5.5, 6.0 and 6.5; Tris/acetate, pH 7.0, 7.5, 8.0 and 9.0.

lactose (3'SL) and the α2-3-sialylated pentasaccharide, LSTa, was evident, in contrast with the absence of transfer activity when the sialyl α2-6 structural analogues (6'SL and LSTc) were tested as acceptors (Figure 1). Since a comprehensive survey of rat organs indicated that spleen had, to a substantial extent, the highest PAPS:3'SLN-sulphotransferase activity (see section below), we carried out our detailed investigations of this enzyme with this lymphoid tissue.

Properties 3'SLN-sulphotransferase

Enzyme activity towards 3'SLN increased with time for at least 2 h at 30 °C, although there was a departure from linearity by 60 min; no detectable activity was observed in the absence of the exogenous acceptor (results not shown). Furthermore, this sulphotransferase activity increased in a linear manner with the amount of microsomal membranes when assay in the 40–240 µg protein range under standard conditions for 60 min (results not shown).

Optimal transfer activity occurred between pH 6.5 and 7.0, with a sharp decline in both the acidic and alkaline range (Figure 2). Sulphation of 3'SLN was stimulated by manganese with an optimum between 12 and 25 mM, while Mg²⁺, Ca²⁺ and Zn²⁺ had no perceptible effect. In the presence of Co²⁺, however, a stimulation equivalent to 32% of that brought about by Mn²⁺ was observed. The inclusion of Triton X-100 in the incubation resulted in a pronounced stimulation of sulphotransferase action that was already evident at 0.02% (v/v) and levelled off after a concentration of 0.035% (v/v) had been reached (results not shown).

Kinetic measurements with various PAPS concentrations (at 90 min) yielded a sigmoidal curve for which a *K_m* value of 4.7 µM was calculated; an examination of the effect of increasing concentrations of 3'SLN on enzyme activity (at 60 min) gave a *K_m* value of 0.72 mM. For the reasons stated in the Experimental section, these *K_m* values must be considered tentative.

Substrate specificity of sulphotransferase

When fetuin glycopeptides with an N-linked carbohydrate unit in which the oligosaccharide branches contain the NeuAcα2-

Table 1 Specificity of spleen PAPS:3'SLN-6-O-sulphotransferase towards oligosaccharide acceptors

Incubations were carried out with rat spleen microsomes (138 µg of protein) and [³⁵S]PAPS (0.5 µCi) for 90 min at 30 °C with the indicated acceptor (50 nmol; 0.77 mM) under the conditions described in the Experimental section. The products were quantified by liquid-scintillation counting after column-chromatographic and TLC purifications. Acceptor abbreviations are defined in the text.

Acceptor	Relative activity
3'SLN	100
6'SLN	0
3'SL	89
6'SL	0
LSTa	109
LSTc	0
3'SLe ^x	0
DSLNT	0
DSL	0
LacNAc	0†

* Values are expressed relative to 3'SLN, into which 2450 d.p.m. was incorporated during the period of assay.
† TLC indicated that sulphate was incorporated exclusively into Gal(3-SO₄)β1-4GlcNAc.

Figure 3 Effect of neuraminidase ('Neurase') treatment on the TLC migration of ³⁵S-labelled sulphated 3'SLN and LSTa

After purification by preparative TLC, [³⁵S]3'SLN and [³⁵S]LSTa produced by the spleen enzyme were incubated with (+) or without (–) with *Arthrobacter ureafaciens* neuraminidase as described in the Experimental section and subsequently chromatographed on a cellulose-coated plate in solvent system B for 46 h (left side) or 90 h (right side). The components were detected by fluorography.

3Galβ1-4GlcNAc sequence [30] were tested as acceptors, effective transfer of sulphate took place and an approximate *K_m* value of 1.16 mM was determined.

An examination of a number of acceptors indicated that the sulphotransferase acted only on oligosaccharides terminating in sialic acid linked to galactose by an α2-3 bond. Although the GlcNAc in the sialyl-lactosamine sequence could be exchanged for a Glc residue and a Galβ1-3GlcNAc linkage could be substituted for Galβ1-4GlcNAc without much change in activity, the linkage of fucose to the GlcNAc, as in 3'SLe^x, or NeuAc, as in DSLNT, resulted in totally inert acceptors (Table 1). Although

Figure 4 Lectin chromatography on an SNA-agarose column of ³⁵S-labelled sulphated 3'SLN before and after neuraminidase treatment in comparison with other sulphated disaccharides

Purified sulphated 3'SLN ([³⁵S]3'SLN) before and after neuraminidase digestion 'Neurase' were chromatographed on an SNA-agarose column as described in the Experimental section. After application of the sample the column was eluted with 0.15 M NH₄HCO₃ containing 0.1 mM calcium acetate. Fractions (1 ml) were collected and monitored for radioactivity by liquid-scintillation counting. For comparison, ³⁵S-labelled Gal(3-SO₄)β1 → 4GlcNAc {[³⁵S]Gal(3S)β1 → 4GlcNAc} and GlcNAc(6-SO₄)β1 → 2Man {[³⁵S]GlcNAc(6S)β1 → 2Man} as well as [³H]galactose-labelled Galβ1 → 4GlcNAc(6-SO₄) {[³H]Galβ1 → 4GlcNAc(6S)} were chromatographed in the same manner. The preparation of these radiolabelled sulphated disaccharides is described in the Experimental section. The efficacy of the column was confirmed by the finding that NaBH₄-reduced 3'SLN passed unretarded through the column, while the 6'SLN standard was retained.

desialylated fetuin glycopeptides and LacNAc incorporated radioactivity, the [³⁵S]sulphate was found to be exclusively located on C-3 of the terminal galactose residue, and this was attributed to the action of the previously described [14] PAPS:galactose 3-O-sulphotransferase that is also present in spleen microsomes (see section below).

Characterization of the sulphotransferase product

Treatment of the chromatographically purified [³⁵S]sulphate-labelled 3'SLN and LSTa with *Arthrobacter ureafaciens* neuraminidase resulted in an increase in the TLC migration (solvent system B) of these two components from *R_{LacH₂}* (chromatographic mobility relative to lactitol) 0.46 to 1.20 and from *R_{LacH₂}* 0.13 to 0.30 respectively (Figure 3). As no radiolabel was lost by this

Figure 5 Effect of periodate oxidation on the sulphated disaccharide obtained after neuraminidase digestion of ^{35}S -labelled 3'SLN

The disaccharide resulting from neuraminidase treatment of ^{35}S 3'SLN (see Figure 3) was examined by TLC with (+) or without (–) undergoing prior Smith periodate degradation (NaIO_4). The standard (STD) α -glycerol [^{35}S]sulphate [Gly(S)] was prepared in the manner described in the Experimental section. Chromatography was carried out on a cellulose-coated plate in solvent system B for 24 h. Components were revealed by fluorography; the arrow indicates the migration of standard galactitol.

treatment, it was evident that the sulphate group was located internally to the sialic acid on either the Gal or GlcNAc residues.

Since it has been reported that SNA binds 6'SLN but not the 3'SLN trisaccharide [31] and, moreover, that LacNAc with a sulphate substitution on C-6 of the galactose [i.e. Gal(6- SO_4) β 1-4GlcNAc] also reacts with this lectin [32], we employed immobilized SNA to explore the position of the sulphate on our ^{35}S 3'SLN product. Chromatography on SNA-agarose of the ^{35}S sulphate-labelled 3'SLN both before and after neuraminidase treatment indicated that, although the native product did not bind to the column, removal of the α 2-3-linked sialic acid residue yielded a disaccharide which was bound to the lectin (Figure 4). This stood in contrast with the finding that a number of other sulphated disaccharides, including Gal(3- SO_4) β 1-4GlcNAc, did not interact with the lectin. These observations, together with the knowledge that the OH group at C-3 and C-4 of galactose are a requirement for SNA interaction [33], suggested that our radiolabelled 3'SLN was sulphated at the C-6 position of Gal.

Consistent with this structural assignment of the sulphate group was our observation that the neuraminidase-treated ^{35}S sulphate-labelled 3'SLN yielded upon Smith periodate degradation a radiolabelled component which migrated on TLC to the position of α -glycerol [^{35}S]sulphate (Figure 5). This finding excluded the possibility that the sulphate group was situated on the GlcNAc residue or the C-3 position of the galactose component, as such locations would have imparted resistance to the periodate oxidation [34]. Moreover, C-4 sulphation of galactose would have yielded radiolabelled threitol sulphate [34]. The possibility that the sulphate is located on C-2 of galactose cannot be excluded, as the β -glycerol sulphate resulting from that situation would unlikely separate from the α -glycerol sulphate on TLC; however, in view of the SNA chromatographic data, this alternative possibility to a C-6 substitution seems unlikely.

Treatment of the ^{35}S sulphated fetuin glycopeptides by the hydrazine/ HNO_2 / NaBH_4 procedure provided further infor-

Figure 6 TLC examination of the ^{35}S -labelled product obtained by hydrazine HNO_2 / NaBH_4 treatment of sulphated fetuin glycopeptides

The purified ^{35}S -labelled products resulting from the action of spleen microsomal sulphotransferase on native sialylated (Nat) and desialylated (Neu-free) fetuin glycopeptides were submitted to the hydrazine/ HNO_2 / NaBH_4 procedure described in the Experimental section. The native ^{35}S -labelled glycopeptides were treated with neuraminidase prior to being submitted to the degradation reaction. Chromatography of the product was carried out on a cellulose-coated plate in solvent system B for 28 h. For comparison, ^3H -labelled disaccharides (STDS) representing Gal β 1 $\rightarrow$ 4AnManH₂(6- SO_4) and Gal(3- SO_4) β 1 $\rightarrow$ 4AnManH₂ were chromatographed in lanes 1 and 2 respectively; the preparation of these saccharides is described in the Experimental section. Components were revealed by fluorography.

mation which was in accord with a NeuAc α 2-3Gal(6- SO_4) β 1-4GlcNAc structural formulation (Figure 6). Degradation of the native sulphated glycopeptide by this approach, subsequent to its desialylation with neuraminidase, yielded a radioactive component, most likely Gal(6- SO_4) β 1-4AnManH₂ (AnManH₂ is 2,5-anhydromannitol), which migrated to a position ($R_{\text{LacH}_2} = 1.19$) that was distinguishable from Gal(3- SO_4) β 1-4AnManH₂ ($R_{\text{LacH}_2} = 1.70$) and Gal β 1-4AnManH₂(6- SO_4) ($R_{\text{LacH}_2} = 1.27$), which are the disaccharides obtained as the fragmentation products of Gal(3- SO_4) β 1-4GlcNAc and Gal β 1-4GlcNAc(6- SO_4) sequences respectively [1]. Indeed, when desialylated fetuin glycopeptides were used as substrate with spleen microsomes, hydrazine/ HNO_2 / NaBH_4 treatment of the ^{35}S sulphated product yielded the Gal(3- SO_4) β 1-4AnManH₂ disaccharide (Figure 6) as would be anticipated if the PAPS:galactose-3-O-sulphotransferase [14] was also present in these membranes (see section below).

Tissue distribution of sulphotransferases

Since the identification of a PAPS:3'SLN-6-O-sulphotransferase added a new enzyme to the two previously characterized [14,16] sulphotransferases which are potentially involved in the sulphation of LacNAc and sialyl LacNAc sequences, we undertook a comparative survey of the activities of these three enzymes in various rat tissues (Table 2). These measurements indicated that the activity of the 3'SLN-6-O-sulphotransferase was substantially higher in spleen than in any other rat tissue examined and indeed was conspicuously greater than in the other lymphoid tissues (i.e. thymus and lymph nodes). In contrast, the activity of the Gal-3-O-sulphotransferase was high in each of the three lymphoid tissues and, moreover, substantially greater than in any other organ except brain (Table 2). Spleen along with thymus and lymph nodes manifested substantial GlcNAc-6-O-sulpho-

Table 2 Tissue distribution of sulphotransferases which can participate in the formation of sulphated LacNAc and sialyl LacNAc sequences

Incubations were carried out with postnuclear membranes from male rat tissues and [³⁵S]PAPS (0.5 μ Ci) for 90 min at 30 °C as described in the Experimental section. The acceptors (65 nmol; 1 mM) for the three enzyme activities were 3'SLN, LacNAc and GlcNAc β 1-6Man α 1-O-Me respectively. Several concentrations of enzyme were assayed to assure that the assays were in the linear range.

Tissue*	Relative activity $\dagger$		
	3'SLN-6-O-sulphate $\dagger$	Gal-3-O-sulphate	GlcNAc-6-O-sulphate
Spleen	100	100	100
Thymus	6	179	32
Lymph node	28	281	40
Liver	2	2	28
Kidney	1	30	11
Lung	16	2	58
Brain	45	128	198
Testes	51	2	39
Muscle	1	—§	—§
Heart	1	3	12

* Analysis of rat stomach for 3'SLN-6-O-sulphotransferase gave a relative activity of 3.

$\dagger$ The three enzymes assayed are respectively PAPS:3'SLN-6-O-, Gal-3-O- and GlcNAc-6-O-sulphotransferases. Subsequent to the action of Gal-3-O-sulphotransferase, sialylation does not occur, while the product of GlcNAc-6-O-sulphotransferase is a substrate for the sequential action of galactosyl- and sialyl-transferases.

$\ddagger$ The activities, determined as d.p.m./mg of protein, are expressed relative to those of spleen membranes which incorporated 14206, 108614 and 1983 d.p.m./mg of protein into 3'SLN, LacNAc and GlcNAc β 1-6Man α 1-O-Me respectively during the period of the assays.

§ Activity was not detectable.

transferase activities that were, however, again noticeably exceeded by brain (Table 2).

DISCUSSION

It is apparent from the present investigation that spleen microsome contain an enzyme which serves to transfer sulphate from PAPS to the C-6 position of the galactose residue in oligosaccharides containing the 3'SLN sequence. The specificity of this PAPS:3'SLN-6-O-sulphotransferase indicates that it is well suited to participate in the assembly of carbohydrate units in which either 3'-sialyl-6'-sulpho-LacNAc [NeuAc α 2-3Gal-(6-SO₄) β 1-4GlcNAc] or 3'-sialyl-6'-sulpho-Lewis^x [NeuAc α 2-3Gal-(6-SO₄) β 1-4(Fuc α 1-3)GlcNAc] saccharide constellations occur. Indeed our observation that this sulphotransferase acts exclusively on oligosaccharides which are capped by an α -2,3-linked neuraminic acid residue is consistent with the *in vivo* studies that indicate that sialylation precedes sulphate addition on C-6 of galactose [11] and differs from the synthetic order for the assembly of NeuAc α 2-3(6)Gal β 1-4GlcNAc(6-SO₄) sequences in which both galactosylation and sialic acid addition follow the sulphation step [16]. The inactivity of the spleen enzyme towards a 3'-sialyl Lewis^x acceptor suggests that fucosylation is the last step in the formation 3'-sialyl-6-sulpho Lewis^x and is consistent with a scheme proposed by Chandrasekaran et al. [35]. Our finding that C-6 sulphation of 3'SL was almost as effective as that of 3'SLN has an intriguing parallel in binding studies that have indicated that substitution of glucose for GlcNAc does not impair the interaction of 3'-sialyl Lewis^x with L-selectin [24]. Furthermore, the comparable activity which the spleen enzyme exhibited toward NeuAc α 2-3Gal β 1-3GlcNAc and NeuAc α 2-3Gal β 1-4GlcNAc acceptors seems to mirror the finding that both 3'-sialyl Lewis^x and 3'-sialyl Lewis^a can serve as ligands

for the selectins [20,36]. Since the tentative K_m values for 3'SLN and glycopeptides containing N-linked carbohydrate units with NeuAc α 2-3Gal β 1-4GlcNAc branches were quite similar (0.72 mM and 1.16 mM respectively), it would appear that the determinants for the newly described sulphotransferase are primarily located in the peripheral trisaccharide sequence of the acceptors. In accord with this contention is our observation that 3'SLN manifests about the same acceptor activity as the LSTa pentasaccharide.

A survey of various rat tissues indicates that the distribution of the PAPS:3'SLN-6-O-sulphotransferase is quite selective, with spleen, brain and testes demonstrating high activity, while the enzyme was essentially absent from liver and kidney as well as muscle and heart. Tissues primarily devoted to the production of mucins, like lung and stomach, had low to minimal activity. Particularly revealing was the finding that, of the three lymphoid tissues analysed, spleen had substantially greater activity than either thymus or lymph nodes. It is reasonable to assume that the 3'SLN-6-O-sulphotransferase observed in the latter tissue would be involved in the assembly of the 6'-sulpho Lewis^x groups which have been shown to cap many of the oligosaccharide chains of GlyCAM-1 [25]. This glycoprotein, which is present in mouse lymph nodes, is known to be a ligand for L-selectin [11] and in that capacity its sulphated carbohydrate units are believed to mediate the initial adhesive interaction of lymphocytes with high endothelial venules during the homing process [24]. The particularly high 3'SLN-6-O-sulphotransferase activity of spleen suggests that comparable selectin ligands may be present in that tissue. Although spleen is the predominant organ involved in lymphocyte recirculation, surpassing the total lymph node mass, the molecular mechanism involved in its interaction with these cells has as yet not been defined [37]. While the presence of C-6-situated sulphate groups on galactose in 3'SLN or 3'-sialyl Lewis^x sequences is of considerable biological interest in view of their role as selectin ligands, such anionic substituents may also be present in a variety of other as-yet-uncharacterized glycoproteins. Indeed it has been reported that the NeuAc α 2-3Gal(6-SO₄) β 1-4GlcNAc sequence occurs in the N-linked carbohydrate units of a recombinant human tissue plasminogen activator expressed in mouse epithelial cells [38].

Enzymes involved in the assembly of N- or O-linked oligosaccharides are usually not limited in the distribution to one cell type nor involved exclusively in the biosynthesis of a specific glycoprotein [39]. This generalization is supported by our survey of the tissue distribution of two other, previously described [14,16], enzymes which play a role in sulphating LacNAc and sialyl LacNAc sequences (Table 2). The high activity in lymphoid tissues of PAPS:Gal-3-O-sulphotransferase, an enzyme which was first detected in calf thyroids [14], could reflect to a certain extent the formation of capping Gal(3-SO₄) groups that have been implicated as ligands in selectin interactions [17,20]. Likewise, the PAPS:GlcNAc-6-O-sulphotransferase, which was found to be present in substantial amounts in spleen, thymus and lymph nodes, may participate in the assembly of NeuAc α 2-3Gal β 1-4GlcNAc(6-SO₄) and NeuAc α 2-3Gal β 1-4(Fuc α 1-3)-GlcNAc(6-SO₄) saccharide constellations which, on the basis of binding studies, are believed to be involved in interactions with selectins [24] and have indeed been shown to be present as L-selectin ligands in GlyCAM-1 [11]. It would appear likely that the three sulphotransferases surveyed (Table 2) acting in concert with a number of glycosyltransferases are responsible for the assembly of the sulphated selectin ligands of lymphoid tissues as well as the carbohydrate units of a variety of other glycoproteins. The ultimate biological activity imparted to glycoproteins by the anionic oligosaccharides is most likely dependent not only on the

detailed structure of the saccharide groups, but also on their disposition on the polypeptide chain [19,39].

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